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Adoptive immunotherapy, the in vitro activation and infusion of patient T cells, is a potentially useful immunotherapeutic strategy, but its effectiveness is limited by the poor trafficking and tumor localization of the infused cells. This is caused in part by the trapping and damage of many of the cells during their initial passages through pulmonary microvasculature. Trapping is promoted by the complex adhesive appendages which are characteristic of T cells activated in vitro. We now report that pulmonary trapping of activated T cells was reduced, and their tumor homing to s.c mammary tumors was increased, by rendering them temporarily smooth and nonadhesive. This was accomplished through treatment with ML-7, an reversible inhibitor of myosin function. ErbB2-specific T cells were labeled with fluorescent dye and tracked after infusion into mice bearing the ErbB2-expressing tumor D2F2/E2. ML-7-treated cells showed an 8-fold reduction in 30-min pulmonary localization, relative to controls. Localization in s.c D2F2/E2 tumors increased fourfold at 24 and 48 hr. T cell-mediated anti-D2F2 cytotoxicity in vitro was transiently depressed by ML-7 treatment but recovered fully within 24 hr. The results provide the first validation of the concept that appropriate cytoskeletal alterations can improve T cell localization in tumors after adoptive transfer.

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## INTRODUCTION

Relapse after surgery and chemotherapy is a major cause of therapeutic failure in breast cancer patients. Vaccination against tumor antigens is a strategy which holds great promise for preventing recurrence, but it requires an intact immune system [1]. Immunotherapy by adoptive transfer of activated T cells is potentially an excellent way to prevent local and metastatic tumor growth during the period of immunological depletion which follows intensive radiotherapy and chemotherapy [2]. Patient T cells are stimulated in vitro to a peak of population size and anti-tumor activity and infused back into the patient. A serious limitation on adoptive immunotherapy is the poor ability of the infused T cells to circulate properly. A contributing factor to this problem is the morphological and behavioral phenotype of the cells. T cells normally circulate in a state of rest or early activation, in which they are round, smooth, and nonadhesive. Activation in vitro pushes them into a polarized, spiky, adhesive and highly motile form which they never normally achieve until after extravasation. When infused in this polarized state, T cells tend to clump together and to lodge in microvasculature. Many are trapped, embolized, and damaged during their initial passages through pulmonary microvasculature, and are later destroyed in the liver and spleen. Relatively few transferred cells survive to enter tumor masses and secondary lymphoid organs [3]. The polarized, adhesive phenotype of activated T cells is created by the actin-myosin cytoskeleton, which, in activated T cells, is programmed to produces appendages and to hold adhesion receptors in an activated state [4,5]. We have found previously that activated T cells can be rendered temporarily round, smooth, and nonadhesive in vitro by treatment with pharmacological inhibitors of myosin function (Fig.1 A-C, and unpublished finding). We hypothesized that pretreatment of activated T cells with myosin inhibitors prior to infusion will allow the cells to avoid initial trapping in lung microvasculature and circulate widely before regaining the polarized, tissue-invading phenotype. We have now completed the first steps of testing this overall hypothesis in a murine model of breast cancer. In experiments in which tumor-specific T cells were fluorescently labeled and tracked in vivo, T cells treated with a myosin inhibitor did show significantly lower initial lung localization and greater longer-term localization in tumors. Furthermore, the proliferative and cytotoxic activity of the cells rapidly recovered from the inhibition of myosin function.

## BODY:

*Note: The Aims and Tasks referred to below are not those of the original Statement of Work but rather the revised Statement of Work approved January 2002.*

*Aim 1: To test the hypothesis that temporary depolarization of activated T cells by pharmacological myosin inhibitors will increase the percentage of cells localizing in tumor masses and secondary lymphoid organs after adoptive transfer.*

This was to be accomplished in

**Task1: "Determine effects of myosin-inhibiting drugs on *in vivo* T cell localization."**

***Task1 a and b: . Immunize mice; obtain and specifically expand ErbB2-specific T cell populations.***

Task 1 a and b METHODS.

The procedure for producing murine T cells specific for human ErbB2 specific was developed by a colleague, Wei-Zen Wei, who provided all required cell lines The tumor used in this study was D2F2/E2, a mammary adenocarcinoma which arose from a spontaneous mammary hyperplasia of a BALB/C mouse and which was stably transfected to express full-length human ErbB2 [6]. Syngeneic BALB/c mice received bilateral s.c. flank injections of 5 X 10(5) irradiated D2F2/E2 cells. This was repeated 4 weeks later, and after an additional 4 weeks draining lymph nodes were excised. ErbB2-specific lymphocytes from these nodes were specifically expanded by culture on irradiated cells from a syngeneic fibroblastoid cell line engineered to stably express human ErbB2 as well as K(d), IA(d)αβ, and the costimulator B7.1 [7]. This stimulator line will be referred to as "3T3/E2". The T cells were thinned and fed every 2 days with DME containing 10%FCS, 5U IL-2/ml and 10 ng/ml IL-7, and restimulated with irradiated 3T3/E2 every 14-20 days.

Task 1 a and b RESULTS:

After two rounds of stimulation, the lymphocyte population reached a stable phenotype of >98% CD3+, of which typically 55-60% were CD4+, and 45-50% CD8+. After 3 simulations, lymph node cells from 12 mice could be expanded to up to 1.2 X 10(8) cells. They were used in experiments from 7 - 10 days after their last stimulation. At this point the cells typically exerted approximately 50% specific cytotoxicity against D2F2E2 at a an E:T ratio of 50:1, 35% at 25:1, and 10% at ratio of 10:1, as determined by chromium release assay (not shown). Cytotoxicity vs. the unrelated syngeneic mammary tumor 410.4 was <5%.

***Task 1c. Use fluorescent cell-tracking techniques to determine effects of myosin inhibiting drugs on localization of T cells in mice bearing subcutaneous tumors.***

Task 1c METHODS:

1. TUMOR GROWTH. D2F2/E2 tumors were initiated by s.c. injection of 5 X 10(5) cells in the flanks of Balb/c female mice age 3 - 6 months. Tumor hosts were employed in experiments 3 - 4 weeks later, when their tumors reached approx. 10 mm diameter (0.4 - 0.6 g).

2. CELL LABELING. The nonspecific green fluorescent tracking dye CFSE ("Cell Tracker", Molecular Probes) was used to label T cells before infusion into mice. In pilot studies it was determined that a concentration of 1 uM conferred sufficient fluorescent brightness to track the cells for at least 48 hr while not reducing the ability of the cells to exert cytotoxicity or to migrate into collagen matrix in vitro (not shown).

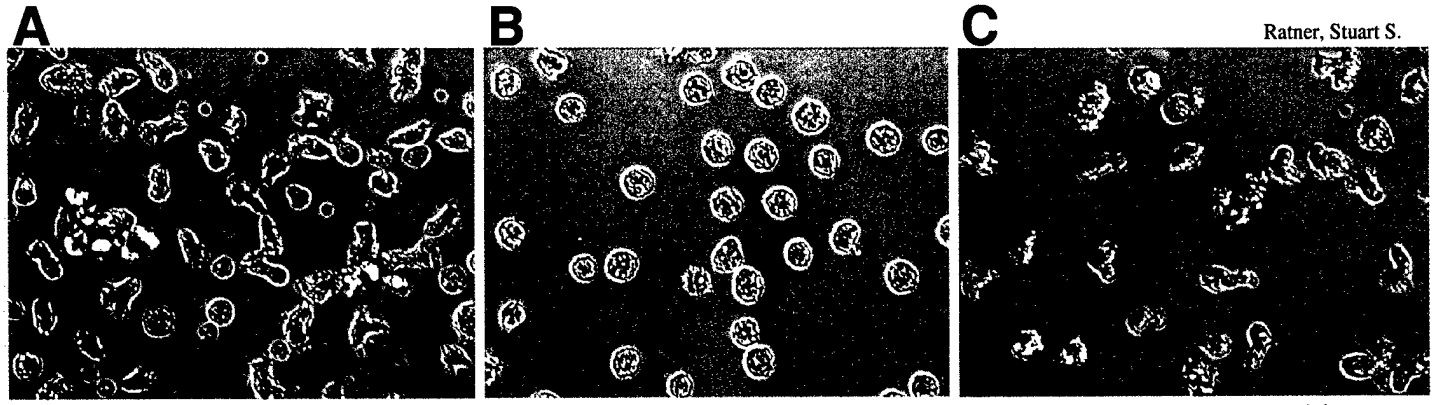
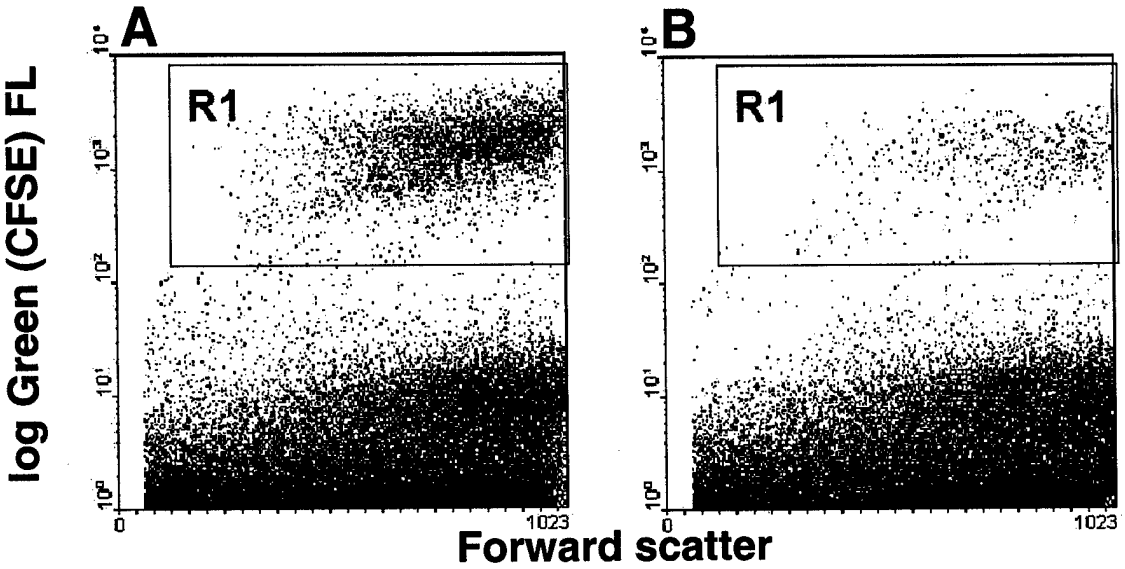



**Figure 1.** Morphological effects induced in activated anti -D2F2/E2 T cells by 20 min. treatment with myosin light-chain kinase inhibitor ML-7 (30 uM). A. Prior to treatment. Note polarized morphology and frequent cell aggregates (e.g. at arrow). B. Immediately after treatment and drug wash-out. Note smooth, spherical morphology and lack of aggregation. C. Two hours after drug wash out. Bar = 10 um.



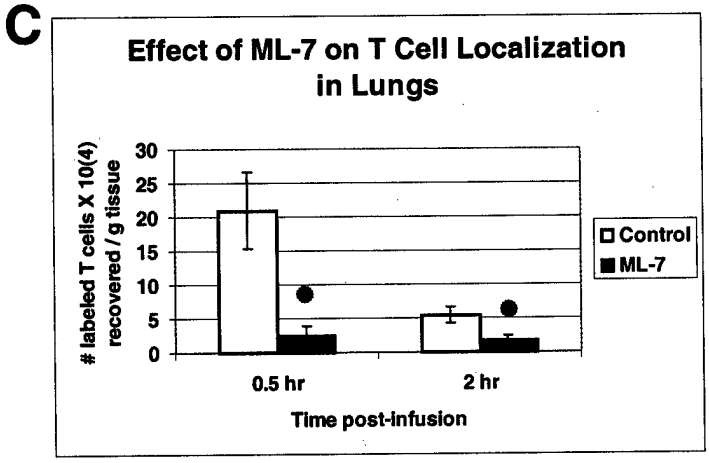




**Figure 2.** Short-term localization pf CFSE-labeled T cells in lungs of Balb?C mice. **A and B.** Typical flow cytometric analyses of single-cell suspension derived from lungs at 0.5 hr after infusion of 10(7) labeled cells. **A.** Control-treated T cells. **B.** T cells treated 20 min with 30 uM ML-7. R1 = region of high fluorescence determined to be unique to viable labeled T cells. C. Summary of data from entire series of experiments, expressed as number of labelled cells recovered per unit tissue weight.
Asterisk = significantly different from control $p < 0.05$.

3. TRACKING EXPERIMENT DESIGN. T cells were labeled by 30-min incubation with CFSE as above and washed 3X with PBS 1% calf serum (CS). They were then treated with myosin inhibitor (see RESULTS for details) or vehicle control, washed 3 more times, the last time in serum-free (SF) Hanks Balanced Saline Solution (HBSS) and injected i.v. at 10(7) cells per mouse in 0.5 ml HBSS. After periods of 0.5, 2.0, 24, or 48 hr, mice were euthnanized by cervical dislocation and tissues harvested for analysis of localization. A total of 6 experiments were run, each with two mice at each time point, one receiving ML-7 treated T cells, and one receiving T cells treated with solvent control (0.001% DMSO ). The mice were drawn from 5 different cohorts of tumor hosts. The exception to this design was an initial series of pilot studies (Task 1c Results, below) in which tissues were harvested from individual mice at 24 hr only.

4. ANALYSIS OF T CELL LOCALIZATION. The following tissues were collected: tumors, peripheral lymph nodes (inguinal, axillary, cervical, mesenteric), spleen, and liver. They were weighed to the nearest mg. Single-cell suspensions were prepared as follows. LYMPH NODES were pressed through wire mesh. SPLEENS were pressed through wire mesh and the resulting suspension was enriched for viable lymphoid cells by centrifugation over Ficoll-Hypaque cushions. LIVERS were minced with a scalpel and incubated at 37°C with constant shaking in an enzyme cocktail until a uniform suspension was obtained (usually 2-3 hr). The cocktail consisted of Liberase Blendzyme 2 collagenase mixture (Roche), 1 U/ml; elastase II (Roche), 2 U/ml, DNAse (Sigma), 3 KU/ml, and 2% CS, all in phosphate-buffered saline (PBS). The resulting cell suspension was filtered through a 60 uM mesh Nitex screen. TUMORS were treated like livers but in addition were centrifuged over Ficoll-Hypaque to deplete nonviable cells.

It was recognized that these dispersal steps caused the loss of an unknown percentage of labeled cells, but there was no reason to believe that the losses were not random. It was therefore assumed that the losses did not bias the comparisons of localization of treated vs. control T cells within each tissue type.

The single -cell suspensions were fixed in 3% paraformaldehyde, washed, and resuspended in a known volume of PBS. A precisely measured aliquot, usually 600 ul, was analyzed by flow cytometry (Becton-Dickinson FACSCalibur). Data were collected through forward and side-scatter gates which excluded nonviable lymphoid cells. In two-dimensional dot plots (forward scatter vs. green fluorescence), labeled T cells appeared in a discrete region which was clearly separated from all unlabeled cells by at least a log of green fluorescence level (e.g. region "R1" in Fig 2a). This region was determined to be empty when only unlabeled cells were infused (not shown). The number of labeled cells in the sample was determined by the analysis function of WinMIDI cytometric software. The number of labeled cells recovered per gram of original tissue was calculated as follows.

$$\frac{(\#\text{ labeled cells per 600 ul cell suspension}) \times (\text{total volume cell suspension})}{(\text{tissue weight in g})} = (\#\text{ labeled cells per g tissue})$$

Statistical analysis was done by paired t test.

Task 1c RESULTS

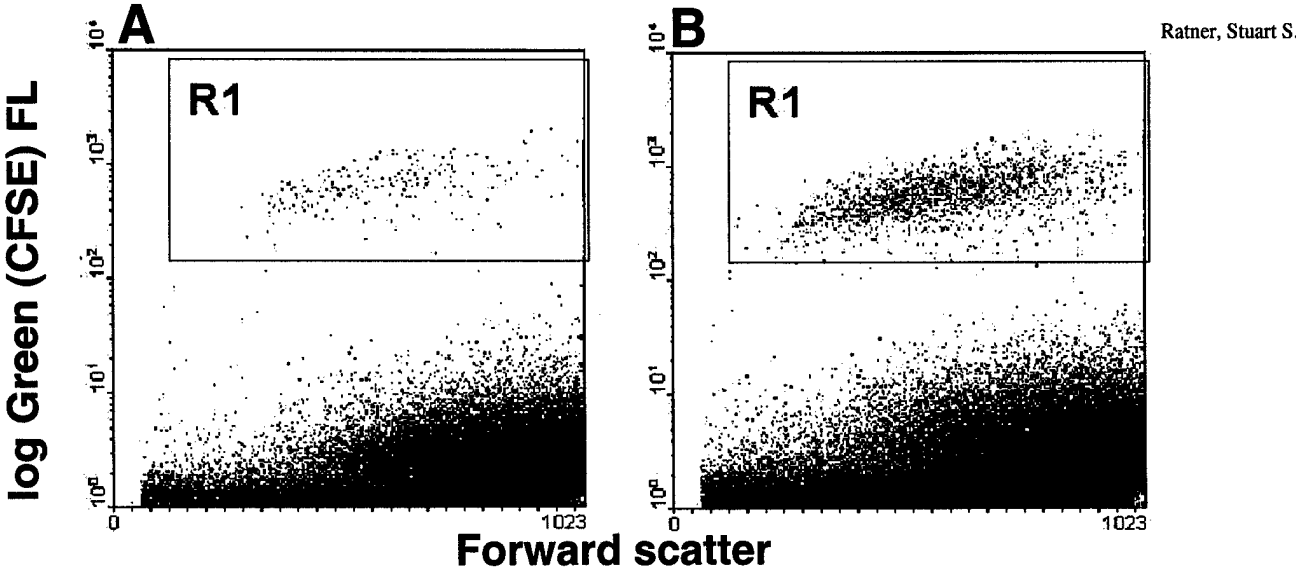
1. PILOT STUDY: OPTIMIZATION OF MYOSIN INHIBITION STRATEGY. Our preliminary results indicated that inhibitors of myosin function synergized to produce in activated T cell populations a period of depolarization and loss of adhesiveness to fibronectin, collagen, and rICAM-1 When used individually, in 20 minute incubations followed by wash-out, the myosin light chain kinase inhibitor ML-7 (Calbiochem, 30 um) and the myosin ATPase inhibitor butanedione monoxime (Sigma, 15 mM) each produced a period of depolarization of 1 - 2 hr, without measurable loss of viability. When used in combination, these two reagents synergized to produce longer-lasting depolarization, up to 12 hr, again without loss of viability. In the pilot tracking experiments of the current project, however, we found that when this combined treatment was used, almost no labeled cells could be recovered from any tissue (not shown). The most likely explanation was that, although harmless in a static culture-dish environment, the dual-drug treatment rendered the cells too fragile to survive the rigors of circulation. The treatment was scaled back to a 20 min incubation with the single agent ML-7 at 30 uM, which produced period of depolarization and loss of adhesion which lasted a mean of 100 min. Fresh resting lymph node cells treated with 30 uM ML-7 homed to lymph nodes at approximately the same rate as did sham-treated controls, indicating that the treatment did not induce mortality after i.v. infusion (not shown).

2. ALTERATION OF TRAFFIC BY PRETREATMENT OF T CELLS WITH MYOSIN INHIBITOR.

***a. Decreased lung localization by treated cells.***

When control cells were infused, their mean 30 min localization in the lungs was 2.1 X $10^5$ cells/g ( $\pm$ 0.57 X $10^5$). In contrast, the treated cells showed more than an eight-fold decrease in localization ($p < 0.05$) (Fig. 2C, and see Fig 2A,B for examples). At 2 hr, about 75% of the control cells had left the lung, but ML-7-treated cells still showed a significant (3-fold) lower localization (Fig. 2C). By 24 hr, virtually no consistently measurable population of labeled cells was recovered from lungs. (not shown).

We have not yet performed histological analysis of the tissue distribution of the labeled cells -- this is planned for Year 2 of the project -- but it is most likely that the heavy 30 min localization of infused control-treated T cells in the lung represented not extravasation but trapping within the microvasculature. The observed kinetics -- prompt accumulation and rapid clearance -- are typical of such trapping (e.g. [8]). The most likely interpretation of our results is that the round and nonadhesive ML-7-treated cells slipped more easily through the lung microvasculature than did the spiki and adhesive control cells. The alternate interpretation is that the ML-7-treated cells were more fragile than the controls and simply ruptured during passage through the lung. This interpretation is extremely unlikely, however, in view of the increased localization of ML-7-treated T cells in tumor and lymph nodes, below.



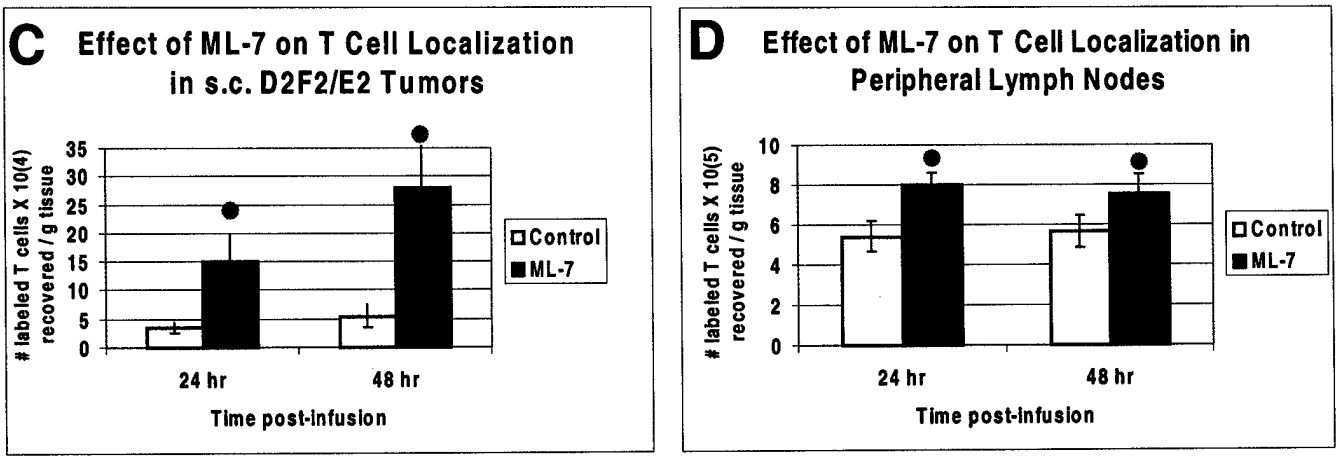




**Figure 3. A-C.** Localization of CFSE-labeled T cells in s.c. D2F2/E2 tumors. **A and B.** Typical flow cytometric analyses of single-cell suspensions of tumor at 24 hr after infusion of 10(7) labeled cells. **A.** Control-treated T cells. **B.** T cells treated 20 min with 30 uM ML-7. R1 = region of high fluorescence determined to be unique to viable labeled T cells. **C.** Data summary from entire series of experiments. Asterisk = significantly different from control $p < 0.05$. **D.** Localization of labeled cells in pooled sets of peripheral lymph nodes. Details as in **C**.

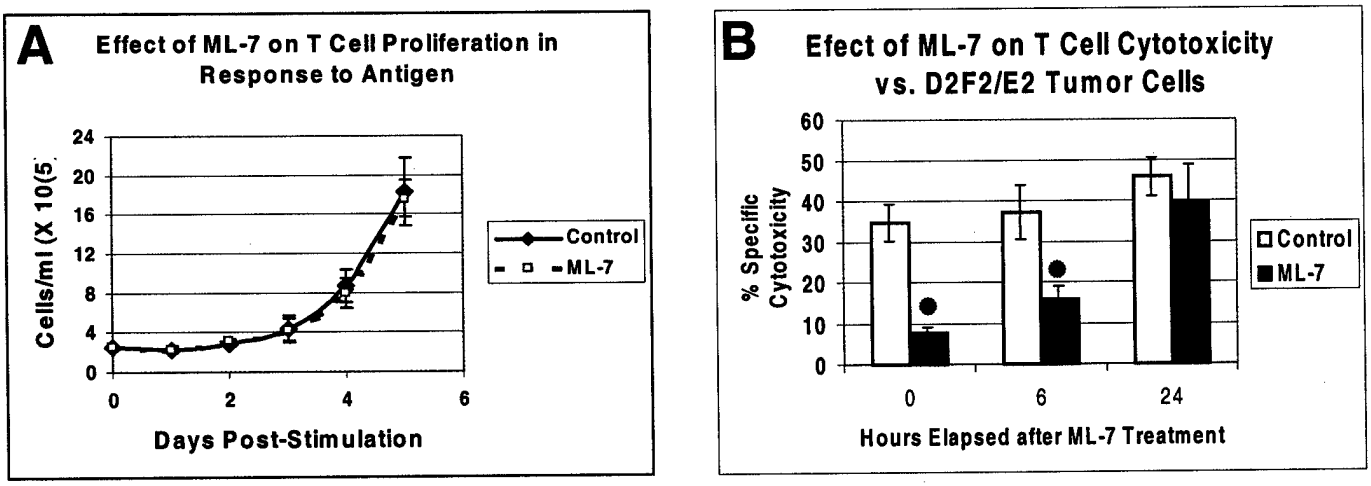



**Figure 4.** Recovery of anti-D2F2/E2 T cell function after transient depolarization by ML-7. **A.** Proliferation during incubation with irradiated stimulator cells. **B.** Cytotoxicity vs. target tumor cells at E:T = 25:1. Asterisks = significantly different from control $p < 0.05$.

***b. Increased tumor localization of treated cells.*** No labelled cells were recovered from s.c. D2F2 tumors at 0.5 or 2 hr, but at 24 hr, mean localization of control T cells was 3.5 X $10^4$ cells/g ( $\pm$ 0.9 X $10^4$). . This represents approximately 0.5% of infused cells and is generally in line withwhat has been reported for other adoptive transfer experiments. ML-7 treated cells showed a greater than 4-fold increase in tumor localization, a mean of 1.5 X $10^5$ cells/g ( $\pm$ .47 X $10^5$) ($p < 0.05$) (Fig 3C and for examples see Fig. 3 A and B). At 48 hr, the longest period attempted, localization of control cells increased slightly, to 5.5 X $10^4$ cells/g ( $\pm$ 2.0 X 10), and treated cells still showed an approximately 4-fold increase ($p < 0.05$) (Fig 3C). The most likely interpretation of the results is that the greater survival of cells during passage through microvasculature led to a larger pool of cells available to localize in tumor.

***c. Increased lymph node localization of treated cells.*** During activation, lymphocytes usually undergo a prompt loss of homing receptors for lymph node endothelium, but some of the cells regain these receptors during further maturation. These lymph-node-homing cells may be important in establishing longer-term immunity after adptive transfer [9]. We found that mean 24 hr localization of labelled control T cells in a set of peripheral lymph nodes was 5.4 X $10^5$ cells/g ( $\pm$ .0.8 X $10^5$). ML-7 treatment increased this localization slightly but significantly to 7.9 X $10^5$ cells/g ( $\pm$ .0.8 X $10^5$) (Fig. 3D). The levels stayed approximately the same at 48 hr (Fig 3D).

***d. Other localizations.*** For control cells, localization in spleen was 4.5 X $10^5$ cells/g, and in liver 6.6 X $10^4$ cells/g. Treated cells showed no significant differences in localaization at these sites (not shown).

***Task 1D. Use fluorescent cell-tracking techniques to determine effects of myosin inhibiting drugs on localization of T cells in mice bearing experimental pulmonary metastases This work is planned for Year 2 of the project.***

*Aim 2 This Original Aim was eliminated from the approved Revised Statement of Work due to the decision to fund two rather than three years of work.*

*Aim 3 To determine the effects of transient depolarization on immunotherapeutically important properties of antigen-specific T cells, including proliferative, cytolytic, and secretory response. This was to be accomplished in*

**Task 2. "Determine effects of myosin-inhibiting drugs on immunotherapeutically important functions of activated T cells *in vitro*" (Months 6-24)**

***TASK 2A . Determine effects of myosin-inhibiting drugs on proliferation in response to antigen stimulation.***

Task 2a METHODS. T cells were cultured as described in Task 1 a and b, above. Two weeks after their last stimulation, when proliferation had essentially ceased, groups of 10(6) T cells were treated 20 min with either 30 uM ML-7 or with 0.001% DMSO vehicle control. The cells were then washed and placed on irradiated 3T3/E2 stimulator cells and culture continued as described in Task 1 a and b, above. Their numbers were followed for the ensuing 5 days This experiment was repeated three times with different batches of T cells.

Task 2a RESULTS

As seen in Fig 4A, there was no significant difference in cell proliferation at any time point. If there was a transient period of unresponsiveness for the ML-7-treated cells, it may have been masked by the normal lag period which the control cells showed before beginning to proliferate

***TASK 2B. Determine effects of myosin-inhibiting drugs on specific cytotoxicty.***

Task 2b METHODS. The cytolytic activity of T cells against the target line D2F2/E2, i.e. the same line used to generate tumors in vivo, was tested in standard chromium release assays. Briefly, D2F2/E2 cells were labeled with $Na^{51}CrO4$ (Perkin Elmer Life Sciences), washed throroughly, and dispensed into 96-well round-bottomed plates, at $10^4$ cells per well. T cells cultured as in Task 1 a and b were tested on days 7 - 12 after their last restimulation. They were divided into two groups, one receiving a 20 min treatment with 30 uM ML-7 and one receiving DMSO vehicle. After three washes, the T cells were returned to culture. In order to measure the effects of ML-7 both immediately after treatment and after various recovery periods, pairs of treated and control cultures were prepared 24 hr, 6 hr, and 0 hr before assay. The T cells were then washed and dispensed into wells at effector to target ratios of ranging from 12.5:1 to 50:1. These will be referred to as "test wells". After 4 hr, a 50 ul sample of supernatant was withdrawn from each well, mixed with 150 ul Optiphase Supermix water-soluble scintillation cocktail, and released isotope was quantitated by beta counting (Trilux MicroBeta Scintillation Counter), with results expressed as cpm. Spontaneous isotope release was determined from supernatants from target wells containing medium only (to be referred to as "medium" wells), and total incorporated isotope was determined from wells in which targets were lysed with 1% Triton X-100 (to be referred to as "total" wells). Three to five replicate wells were run for each condition, and the experiment was repeated three times. Specific cytotoxicity was calculated as follows:

$$\% \text{ specific lysis} = 100 \text{ X } (cpm_{test} - cpm_{medium}) / (cpm_{total} - cpm_{medium})$$

Task 2b RESULTS.

(Note: Results will be reported only for cytotoxicity at an effector : target ratio of 25:1, because in some experiments insufficient cells were available for a complete range of ratios.) When the cytotoxicity assay began immediately after ML-7 treatment,

cytotoxicity against D2F2E2 was only 22% that of control T cells (Fig 4B). At 6 hr post-treatment, the ML-7 treated cells had recovered to 42% the cytotoxicity of controls, and by 24 hr there was no longer a significant difference (Fig 4B).

Other functions Tests of the effects of ML-7 on another immunotherapeutically important function, the elaboration of cytokines, will be part of the second year of Task 2.

## KEY RESEARCH ACCOMPLISHMENTS:

- Short-term inhibition of myosin function was found to reduce the trapping of adoptively transferred T cells in the pulmonary vasculature and to increase their localization in a murine mammary tumor and in lymph nodes.
- Cytotoxic function of and proliferative capability of activated T cells quickly recover from the depressive effects of myosin function inhibition.
- These results provide initial validation of our hypothesis that the homing of immunotherapeutic T cells to tumors can be improved through manipulation of the T cell cytoskeleton.

## REPORTABLE OUTCOMES:

So far, preparation has begun on manuscript: "Diminished pulmonary trapping and increased tumor localization of adoptively transferred T cells after transient depolarization by inhibitor of myosin function."

## CONCLUSIONS:

SUMMARY. The results of the in vivo localization experiments of Task 1 indicate that transient depolarization of activated T cells by a myosin light-chain kinase inhibitor allowed those cells to avoid massive trapping and damage in the pulmonary vasculature after i.v. infusion. The increase in surviving cells apparently led to a significant, fourfold increase in the percentage of infused cells to reach mammary tumors, where they should be able to produce local antitumor effects. There was also greater homing to peripheral lymph nodes, where the T cells may recruit additional effectors and establish long term anti-tumor memory. It is to be expected that disruption of myosin function will disturb a variety of T cell functions. The results of Task 2 experiments performed so far demonstrate that a degree of inhibition sufficient to alter T cell traffic causes only a brief decline in cytotoxic activity and no measurable decrease in ability to proliferate in response to antigen.

IMPORTANCE. The results constitute the first validation of the concept that direct alterations of the T cell cytoskeleton can improve the trafficking of therapeutic lymphocytes to sites of tumor growth. In this case, the manipulation was a simple transient pharmacological inhibition of function of the motor protein myosin, which is

necessary both for the formation of cellular appendages and the maintenance of many types of adhesion receptor in activated configuration. In the future, more specific and controllable cytoskeletal alteration strategies may be developed for pre-infusion treatment of patient T cells. Among the likely possibilities would be antisense RNA against the members of the Rho family of GTPases, which regulate specific features of cellular polarity and motility [10]

"SO WHAT?" Adoptive immunotherapy is in theory an ideal way to provide at least short-term immunosurveillance to breast cancer patients whose immune systems have been debilitated by intensive therapy, but in practice the strategy is little used because of its poor track record in clinical trials. Our findings suggest a way to make adoptive immunotherapy more efficient and may therefore lead to a reconsideration of this mode of treatment.

The findings also have implications for the field of gene therapy. Lymphocytes are being considered as a possible vehicle for carrying genes and gene products into tumors. This strategy too could benefit from a method to make lymphocyte home more effectively to tumors [11].

CHANGES IN FUTURE WORK. In the currently approved Statement of Work, Task 1D is to determine whether myosin inhibition increases the localization of adoptively transferred T cells into experimental pulmonary metastases of the D2F2/E2 tumor. Now that we have found that the treatment markedly increases T cell localization in s.c. tumors, we feel that a more important priority will be to test the immunotherapeutic implications of that heavier localization; that is to do a therapeutic trial comparing infusions of ML-7-treated vs. vehicle-treated T cells in terms of effects on tumor growth and host survival. The revision will be proposed via appropriate channels during the next month.

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